Journal of Medical Virology 60:446–454 (2000)

A Neutralizing Recombinant Human Antibody Fab Fragment Against Puumala Hantavirus

Cristina de Carvalho Nicacio,[1,2]* Åke Lundkvist,[1,2] Katarina Brus Sjölander,[1,2] Alexander Plyusnin,[1,3] Eeva-Marjatta Salonen,[3] and Ewa Björling,[1]

[1]Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden
[2]Swedish Institute for Infectious Disease Control, Stockholm, Sweden
[3]Department of Virology, Haartman Institute, University of Helsinki, Helsinki, Finland

A combinatorial human antibody Fab pComb3H library, generated from splenic lymphocytes of a Puumala hantavirus (PUUV) immune individual, was selected against PUUV using the phage display technique. Panning was carried out with antigens immobilized by MAbs directed to the two PUUV envelope glycoproteins G1 and G2. Thirteen Fabs, with reactivity directed to PUUV and specifically the G2 protein, as assessed by immunofluorescence and ELISA respectively, were isolated in crude preparations. By a focus reduction neutralization test (FRNT), four of the 13 crude Fab preparations exhibited type-specific neutralization of PUUV (strain Sotkamo) with 44–54% reduction in the number of foci. After affinity purification, the four Fab clones exhibited 50% focus reduction of PUUV at concentrations below 2 μg/ml. Sequencing of the heavy and light chain complementarity determining regions (CDR) 1–3 showed that the four selected clones were identical within the antibody binding regions. In inhibition tests with the PUUV G2-specific MAbs, 4G2 and 1C9, a new epitope important for neutralization, designated as G2-a3, was defined. This epitope, overlapping partially the neutralizing epitope recognized by the human MAb 1C9, seems to be unique for the PUUV serotype since none of the Fab clones neutralized any of the other hantaviruses tested. ***J. Med. Virol. 60:446–454, 2000.***

KEY WORDS: phage display; FRNT; G2 protein; hantavirus; Puumala virus; recombinant Fab

INTRODUCTION

Hantaviruses, members of the family *Bunyaviridae*, are known to cause serious and often fatal human diseases, such as hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Each hantavirus is carried primarily by a specific rodent host and transmission from rodents to humans occurs by aerosolized animal excreta [Plyusnin et al., 1996]. Hantaan (HTNV), Dobrava (DOBV), Seoul (SEOV), and Puumala (PUUV) viruses are known to cause different forms of HFRS [Antoniadis et al., 1996; Plyusnin et al., 1996; Lundkvist et al., 1997a]. The clinical symptoms are characterized by fever, renal failure and, in severe cases, hemorrhagic manifestations [Lundkvist and Niklasson 1994; Kanerva et al., 1996]. Clinical manifestations of HFRS are generally most severe for HTNV and DOBV infections whereas PUUV infection is associated with the milder form of HFRS, nephropathia epidemica (NE), occurring in Scandinavia, Finland, Western Russia and Central Europe. The mortality of HFRS varies between <0.1% and 10%, depending on the causative virus, and approximately 100,000–200,000 cases occur annually [Lundkvist and Niklasson 1994; Plyusnin et al., 1996]. The causative agents for HPS are Sin Nombre virus (SNV), and SN-related viruses occurring in North and South America [Nichol et al., 1996; Lopez et al., 1996]. The disease is characterized by acute respiratory distress and has a mortality rate of approximately 50%. Additional unique hantaviruses, not associated with human disease to date, include e.g.: Prospect Hill (PHV), Thailand, Thottapalyam, Khabarovsk (KBRV), Tula (TULV) and Topografov (TOPV) viruses [Hörling et al., 1996; Plyusnin et al., 1994; Vapalahti et al., 1999].

Hantaviruses are enveloped negative-stranded RNA viruses with a tripartite genome with the large (L), medium (M) and small (S) segments packed in helical nucleocapsids [Schmaljohn, 1996]. The genome encodes four structural proteins (sizes given for PUUV):

Grant sponsor: Tobias Foundation; Grant sponsor: Salén Foundation; Grant sponsor: Swedish Medical Research Council; Grant number: 12177, 12642; Grant sponsor: European Community; Grant number: (Biomed-2) BMH4-CT97-2499.

*Correspondence to: Cristina de Carvalho Nicacio, Microbiology and Tumorbiology Center, Box 280, Karolinska Institute, S-171 77 Stockholm, Sweden.

Accepted 4 October 1999

the 6.5 kb L segment encodes the approximately 200 kDa RNA polymerase protein, the 3.7 kb M segment encodes the two glycosylated envelope proteins, G1 (68 kDa) and G2 (54 kDa), and the 1.8 kb S segment encodes the 50 kDa nucleocapsid protein (N) [Plyusnin et al., 1996; Schmaljohn, 1996].

Several monoclonal antibodies (MAbs) have been generated against the G1, G2 and N proteins of PUUV [Lundkvist et al., 1991; 1993; Lundkvist and Niklasson, 1992], and neutralizing epitopes have been defined in both G1 and G2. Most MAb epitopes in the glycoproteins seem to be conformational, as none of the MAbs available currently are reactive in immunoblots carried out on denatured proteins [Hörling and Lundkvist, 1997]. This is probably associated with the complex three-dimensional structure of the glycoproteins, due to a high content (5.4%) of conserved cysteine residues and the multiple glycosylation sites within hantavirus glycoproteins [Plyusnin et al., 1996]. The G2 protein contains MAb epitopes shared by several hantaviruses, whereas the epitopes of G1 seem to be more virus-specific [Chu et al., 1994].

Passive transfer of rat immune sera and rodent MAbs protect rats, hamsters, mice and bank voles from SEOV, HTNV and PUUV infection, suggesting that antibodies alone are sufficient for protective immunity [Zhang et al., 1989; Schmaljohn et al., 1990; Arikawa et al., 1992; Lundkvist et al., unpublished]. Passive immunization for prophylaxis and therapy with neutralizing human MAbs might therefore be of use for controlling human infection.

Combinatorial library technology, i.e., antigen selection from antibody libraries generated by recombinant phages [McCafferty et al., 1990; Barbas et al., 1991], offers an alternative approach to the generation of antibodies. It provides rapid access to large numbers of human MAbs and may be used for the analysis of human antibody responses. PUUV-specific Fabs directed against the N and G2 proteins have been described previously [Salonen et al., 1998]; however, none of these Fab fragments displayed neutralizing capacity in vitro.

In this study, the generation of PUUV-specific neutralizing human Fab fragments is described by panning of a combinatorial Fab library against native viral proteins captured by specific MAbs immobilized on microtiter plates. The technique allows the selection and isolation of recombinant Fab fragments directed against conformational epitopes.

MATERIALS AND METHODS

Antibody Phage-Display Library

A cDNA library of the IgG1 heavy chain Fd regions and whole κ light chains was expressed in the phage-display vector pComb3H, essentially as described earlier [Burton et al., 1991; Persson et al., 1991; Salonen et al., 1998]. The RNA source consisted of splenic lymphocytes from a splenectomized PUUV immune patient [Lundkvist et al., 1993; Salonen et al., 1998]. Human MAbs were previously isolated from EBV transformed B cells from the same donor [Lundkvist et al., 1993], and the Mab 1C9 has been expressed in the baculovirus system [Liang et al., 1997]. The donor serum has been reported previously to have high IgG reactivity against the PUUV nucleocapsid protein and the two envelope glycoproteins [Lundkvist et al., 1993].

Selection of Antibody Binding Clones by Panning

The combinatorial library was panned against PUUV antigens captured by PUUV-specific MAbs. Immunoplates (Nunc, Denmark) were coated overnight with 1 μg/well of bank vole MAbs 5A2 or 5B7 specific for G1 and G2 proteins, respectively, in 0.05 M bicarbonate buffer, pH 9.6, at 4°C. Plates were washed six times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 3% BSA for 1 hr at 37°C. Detergent-treated native PUUV antigen was prepared as previously described [Lundkvist and Niklasson, 1992], diluted in PBS-T containing 0.5% BSA (dilution buffer) and incubated (100 μl/well), at 4°C overnight. To eliminate nonspecific binding, 100 μl of phage resuspended in PBS containing 1% BSA was preadsorbed to wells coated with only MAbs (5A2 and 5B7). Phages were then incubated with antigen for 2 hr at 37°C. Unbound phages were removed by vigorous washing with PBS-T after each round of panning. Bound phages, bearing antigen-binding surface Fabs, were eluted with 0.1 M HCl-glycine, pH 2.2. The eluted phages were amplified by infection of *E. coli* XL1-Blue (Stratagene, La Jolla, CA) cultures followed by superinfection with helper phage VCS-M13 (10^{12} plaque-forming units) (Stratagene). After the fourth round of panning, no helper phage was added and phagemid DNA was prepared from the overnight culture.

Excision of the cpIII gene fragment from purified phage DNA, propagation and induction of Fab production were carried out essentially as described previously [Burton et al., 1991]. The resulting Fab supernatants were screened against detergent-treated PUUV antigen, captured by the anti-G1 and G2 MAbs 5A2 and 5B7 in ELISA.

Purification of Fabs

An affinity gel was produced by linking 2.5 mg goat anti-human IgG $F(ab')_2$ (Pierce, Rockford, IL), to 4 ml Affi-Gel 10 (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. After blocking and washing, the affinity gel was packed into a column. Fabs were prepared from isopropyl β-D-thiogalactopyranoside (IPTG)-induced 500 ml cultures and purified as described previously [Samuelsson et al., 1995]. Purified Fab preparations gave bands of approximately 25 kDa in reducing SDS PAGE.

ELISA

Screening of crude Fab preparations, determination of Fab concentrations, confirmation of Fab antigenic specificities and competition studies were carried out by ELISA. For all assays, the following reagents were

used: antigens or antibodies were diluted in 0.05 M bicarbonate buffer, pH 9.6, and coated to microtiter plates overnight at 4°C. Coated plates were blocked with either 4% dry milk (Bio-Rad) in PBS, or 3% BSA in PBS. All reagents were diluted in dilution buffer containing 0.5% BSA in PBS-T, and the plates were washed six times in PBS-T between each step. All incubations were carried out at 37°C for 1 hr. The assays were developed with either o-phenylenediamine dihydrochloride (OPD) (Sigma, St Louis, MO) or *p*-nitrophenyl phosphate (NPP) (Sigma) in 10% diethanolamine. Absorbances were measured at 490 nm or 405 nm, respectively.

Antigen-capture ELISA for determination of antigen specificity. Plates were coated with the PUUV-specific MAbs 5A2 (anti-G1), 5B7 (anti-G2) or 1C12 (anti-N) at 0.5 μg/well, followed by detergent-treated PUUV antigen [Lundkvist and Niklasson, 1992]. After blocking, undiluted crude Fab supernatants or purified Fabs were added. Bound Fabs were detected with horseradish peroxidase (HRP)-labeled goat anti-human IgG $F(ab')_2$ antibody (Pierce) diluted 1:1000, followed by OPD.

Determination of Fab concentration. For determination of Fab concentrations, goat anti-human IgG $F(ab')_2$ (Pierce) was coated onto microtiter plates. After blocking, sequentially diluted Fab samples and a standard control (Fab fragment of human IgG, 0.0001–1 μg/ml, Nordic Immunology, Tilburg, The Netherlands) were added. Antibody binding was detected with goat anti-human IgG $F(ab')_2$-HRP diluted 1:1000, followed by OPD.

Cross-reactivity with unrelated proteins. The antigenic specificity of the Fabs was evaluated by analysis of the reactivities to tetanus toxoid (SBL Vaccin, Stockholm, Sweden), ovalbumin (Worthington Biochemical Corp., Lakewood, NJ) BSA and measles virus (SMI, Stockholm, Sweden). These antigens (2.5–10 μg/ml) were coated directly on microtiter plates, and the ELISA was conducted as described above.

Competitive binding assays. Three well-characterized PUUV G2-reactive MAbs, the bank vole MAbs 4G2 and 5B7 directed against the G2-a1 and G2-b epitopes, respectively, and the human MAb 1C9 directed against the G2-a2 epitope [Lundkvist and Niklasson, 1992; Heiskanen et al., 1997; Hörling and Lundkvist, 1997] were used in competitive binding assays to determine the epitope-specificities of the Fabs. Cross-competition binding experiments including biotin-labeled and unlabeled Fabs and immobilized G2 protein were also undertaken. Biotinylation of Fabs was carried out as described previously for MAbs [Lundkvist et al., 1993]. MAbs were coated on microtiter plates at 1.0 μg/well, followed by detergent-treated PUUV antigen in dilution buffer. After blocking, inhibiting Fabs were added at a concentration of 20 μg/ml, followed by biotinylated MAbs or Fabs. Specific antibody binding was detected with Extravidin-alkaline phosphatase (Sigma) diluted 1:5000, followed by NPP. Blocking was assessed and compared to controls (non-reactive Fabs, MAbs, PUUV positive sera or diluent only).

Immunoblotting

The reactivity of Fabs with denatured PUUV antigen was examined by immunoblotting. Blotting was carried out essentially as described earlier [Lundkvist et al., 1991]. Detergent-treated PUUV antigen was mixed with SDS sample buffer, applied to a 4–15% gradient SDS-polyacrylamide gel and transferred to a nitrocellulose sheet. Nitrocellulose strips were blocked and incubated overnight with 2 μg/ml Fab at 4°C. After four washes, the strips were incubated for 4 hr at RT with alkaline phosphatase-conjugated goat anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch laboratories Inc., West Grove, PA) diluted 1:1000. Strips were washed as above and developed with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets dissolved in distilled water (Sigma). MAbs reactive against PUUV N protein [4C3 and 1C12, Lundkvist et al., 1991] and a PUUV positive serum were included as controls.

Immunofluorescence Assay (IFA)

Vero E6 cells infected with PUUV (strains Sotkamo, Vindeln 83-L20 and Kazan-E6) [Vapalahti et al., 1992; Hörling et al., 1995; Lundkvist et al., 1997b; Hörling and Lundkvist, 1997], HTNV [strain 76–118, Lee HW et al., 1978], SEOV [strain 80–39, Lee HW et al., 1982], PHV [strain PH-1, Lee PW et al., 1982], DOBV [Avsic-Zupanc et al., 1992], TULV [strain Tula-MA76-1987, Vapalahti et al., 1996], KBRV [strain MF-43, Hörling et al., 1996], TOPV [Vapalahti et al., 1999], or SNV [strain CC-107, Schmaljohn et al., 1995] were fixed onto slides with cold acetone. Crude Fabs or purified Fabs diluted 1:2 (≈12.5 μg/ml) in PBS, were incubated for 2 hr followed by a 1 hr incubation with fluorescein isothiocyanate-conjugated goat anti-human $F(ab')_2$ antibody (Jackson ImmunoResearch laboratories Inc.) at 1:50 dilution for detection of virus-specific antibody binding.

Virus Neutralization Assay

The ability of Fabs to inhibit hantavirus infectivity was assayed by a focus reduction neutralization test (FRNT) [Lundkvist et al., 1997a]. Fabs were diluted serially two-fold starting at 1:2 in Hank's balanced salt solution supplemented with 2% 1 M HEPES, 2% fetal calf serum (FCS) penicillin (50 μg/ml), streptomycin (50 μg/ml) and neomycin (100 μg/ml), and mixed with an equal volume of PUUV (strains Sotkamo, Vindeln 83-L20 and Kazan-E6), HTNV (strain 76-118), SEOV (strain 80-39), PHV (strain PH-1), DOBV, TULV (strain Tula-MA76-1987) or TOPV at 50 focus-forming units (FFU) per 100 μl. The mixture was incubated for 1 hr at 37°C and subsequently inoculated (200 μl) into six-well tissue culture plates containing confluent Vero E6 cell monolayers. After adsorption for 1 hr at 37°C, the wells were overlaid with a 42°C mixture of one part 1% agarose and one part 2× basal Eagle's medium with

L-glutamine and Earle's salts supplemented with 8% FCS, 2.5% 1 M HEPES and antibiotics as above. The tissue culture plates were incubated at 37°C in a humidified 5% CO_2-atmosphere for 7–13 days depending on the virus used. For foci detection, cells were fixed for 8 min with methanol, followed by air drying. Rabbit anti-hantavirus serum diluted 1:300 in dilution buffer (PBS containing 0.1% Tween-20 and 5% FCS), was added to each well and plates were incubated for 1 hr at 37°C. Washed plates were incubated with goat anti-rabbit IgG-HRP diluted 1:1000 for 1 hr at 37°C. After additional washings, foci were visualized with tetramethylbenzidine (TMB) substrate (Sigma). The reaction was stopped with 2 M H_2SO_4 and the foci were enumerated.

Nucleic Acid Sequencing

Cycle sequencing was carried out on phagemid DNA using the PRISM Ready BigDye Terminator Cycle Sequencing kit with Ampli Taq DNA Polymerase FS (Taq-FS; Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, CA). Amplification was carried out using the GeneAmp® PCR System 2400 (Perkin-Elmer, Norwalk, CT). The reaction mix (20 μl) contained 8 μl PRISM premix, 3.2 pmol sequencing primer and 500 ng of DNA. The PCR products were analyzed by use of an ABI377 sequencer [PE/ABI].

RESULTS

Isolation of G2-Specific Fab Fragments by Panning

An IgG1 κ antibody library expressed on the surface of filamentous phages, derived from spleen lymphocyte RNA from a PUUV seropositive donor, was used for selection of PUUV-specific Fabs. To enrich for antigen-specific Fab fragments the library was panned four rounds against native viral antigen immobilized in microtiter wells with PUUV G1- and G2-specific MAbs. Phagemid DNA was prepared from the final round of panning and the gene III fragment was removed by *Nhe*I and *Spe*I enzyme digestion followed by religation. The resulting phagemid was transformed into XL1-Blue *E. coli* cells to produce clones secreting soluble Fab fragments. One hundred clones were randomly picked, and freeze-thawed culture supernatants were screened for reactivity against the G1 and G2 proteins of PUUV in antigen-capture ELISA. Thirteen crude Fab bacterial lysates reacted with the G2 protein, whereas none was reactive against the G1 protein. The thirteen crude Fab preparations were examined further by IFA and FRNT. All preparations were IFA positive when tested against PUUV (strain Vindeln 83-L20) infected cells, and all but one gave a detectable (27–54%) focus reduction in PUUV (strain Sotkamo) FRNT (Table I).

TABLE I. Primary Characterization of 13 Crude Fab Preparations by Immunofluorescence Assay (IFA) and Focus Reduction Neutralization Test (FRNT)*

Fab	IFA[a]	FRNT[b]
PUU-21	+	44%
PUU-22	+	0%
PUU-25	+	39%
PUU-29	+	27%
PUU-31	+	29%
PUU-32	+	46%
PUU-35	+	32%
PUU-37	++	34%
PUU-40	+	34%
PUU-49	+	37%
PUU-89	+	54%
PUU-93	+	39%
PUU-100	+	46%

*Percent reduction in number of foci compared to negative control.
[a]Crude Fab preparations were used undiluted against PUUV (strain Vindeln 83-L20) in IFA.
[b]Crude Fab preparations were diluted 1:2 with PUUV (strain Sotkamo) in FRNT.

Characterization of G2-Specific Fabs

On the basis of a higher neutralizing activity in FRNT, four of thirteen ELISA and IFA positive recombinant Fab clones (PUU-21, -32, -89 and -100) were selected for affinity purification and subsequent testing. Affinity purification using an anti-human IgG $F(ab')_2$ column resulted in approximately 1.5 mg/culture liter of each Fab clone. Affinity purified Fabs reacted with G2 but not with G1 or N protein in an antigen-capture ELISA (Table II). Antigen specificity of the Fabs was verified by non-reactivity (OD < 0.1) against measles virus, BSA, ovalbumin or tetanus toxoid (data not shown). Clones were also tested for G2 binding by titration of equivalent amounts of Fab from the four purified clones. All four clones bound to G2 in a concentration-dependent manner (Fig. 1). None of the clones exhibited any reactivity against PUUV antigen in immunoblots at denaturing conditions, indicating that they recognized a conformational structure in the G2 protein (data not shown).

Reactivities to Various Hantaviruses as Determined by IFA

The affinity purified Fab clones (PUU-21, -32, -89, -100) were tested against a panel of hantaviruses by IFA. All four Fab clones tested were reactive against the Sotkamo, Vindeln 83-L20 and Kazan-E6 strains of PUUV, whereas no reactivity was recorded against TOPV, KBRV, TULV, PHV, SEOV, DOBV, HTNV and SNV viruses, indicating that the purified clones were all PUUV-specific.

Virus Neutralization by Fabs

Purified Fabs were examined for their capacity to block the infectivity of PUUV (Sotkamo, Vindeln 83-L20 and Kazan-E6), HTNV, SEOV, PHV, DOBV, TULV and TOPV. Virus neutralization titers were determined as the reciprocal end-point titer of the Fab fragments to reduce the number of foci by 50% and 80%. Neutralization assays were repeated two to three times for each clone. Negative controls included one measles-specific Fab and one irrelevant Fab (both negative to PUUV as determined by ELISA and IFA).

TABLE II. Fab Reactivity to PUUV Proteins Captured by MAbs in ELISA

	Indirectly coated antigen (capture MAb)		
Antibody	G2 (MAb 5B7)	G1 (MAb 5A2)	N (MAb 1C12)
PUU-21	2.586	0.049	0.110
PUU-32	2.591	0.030	0.090
PUU-89	2.603	0.028	0.096
PUU-100	2.538	0.018	0.092
Neg Fab-7	0.013	0.025	0.074
MAb 1C9 (anti-G2)	2.656	0.040	0.097
PUUV conv. serum* (1:500)	2.230	1.119	1.467
PUUV neg. serum (1:500)	0.125	0.042	0.000

Values are mean absorbance (490 nm) of duplicate samples (background obtained against MAb alone has been subtracted).
*PUUV convalescent serum drawn 4 years post infection.

The four Fab clones chosen for analysis, PUU-21, -32, -89 and -100, exhibited neutralizing titers of 16-256 against PUUV (Sotkamo strain), (Table III) and gave 50% focus reduction of the Sotkamo strain of PUUV at concentrations below 2 μg/ml (Fig. 2). Only a weak neutralizing activity was apparent against the Swedish PUUV strain Vindeln 83-L20 by PUU-21 and -32, whereas Fabs PUU-21, -32, -100 exhibited low neutralizing activity against the Russian PUUV strain Kazan-E6. None of the Fabs displayed any neutralizing capacity against HTNV, SEOV, PHV, DOBV, TULV and TOPV (data not shown).

To investigate if the neutralizing capacity of the Fabs could be enhanced, Fab clone PUU-32 was incubated with 50 μg/ml goat anti-human IgG $F(ab')_2$ (Pierce, Rockford, IL), before incubation with PUUV (Sotkamo strain). No differences in neutralizing activity were found between Fab alone or Fab incubated with goat-anti human IgG $F(ab')_2$.

Fab Competition Studies

Competition ELISAs were run to identify common or differing epitope specificities between PUUV Fabs and MAbs characterized previously. In an assay in which Fabs and MAbs, 4G2 (G2-a1) and 1C9 (G2-a2), reactive with two epitopes defined previously on G2, were used to compete for binding to PUUV G2, very low or no competition was recorded, indicating that the Fabs reacted with antigenic region(s) differing from those recognized by the two defined MAbs (Table IV). In competition studies for PUUV G2 binding using unlabeled and biotin-labeled Fabs and MAbs, blocking among homologous Fab clones ranged from 91.9–93.3% and among heterologous Fab clones from 86.9–96.6%, as compared to blocking with dilution buffer only. This indicated that the Fab clones compete for the same epitope on the G2 protein, determined as G2-a3. In the same assay MAb 1C9 blocked itself completely, whereas it only inhibited Fab binding to G2 with a mean of 36.1% (range 33.7–39) (Table IV).

In competition experiments between biotinylated Fab clones and five PUUV human convalescent sera blocking ranged from 76.8–90.8%, indicating epitope G2-a3 as immunodominant for the human antibody response (Table V).

Nucleic Acid Sequencing of Fab Heavy and Light Chain Variable Regions

The complementarity-determining regions 1–3 (CDR 1–3) of the heavy and κ light chains were sequenced to evaluate whether the isolated Fab fragments represented individual or identical clones. This analysis revealed that the four clones chosen for affinity purification and further testing, PUU-21, -32, -89, -100, all had the same heavy and light chain sequences, indicating that they were all identical in the antigen binding regions. Furthermore, these sequences were identical to those of the G2-specific Fab puuA1 described by Salonen et al. [1998].

DISCUSSION

The use of combinatorial libraries displayed on the surface of filamentous bacteriophage offers an efficient means of obtaining a diverse set of human MAbs from an immune donor [Burton and Barbas, 1994]. In this study, a combinatorial library selected against native PUUV antigen captured in microtiter plates was used for the generation of neutralizing recombinant human Fab fragments specific for the G2 envelope glycoprotein of PUUV.

The antigen used for panning is crucial for the selection of biologically functional antibodies of a particular specificity. For instance, whole viral antigen may result in the selection of antibodies against immunodominant proteins or those proteins present in larger amounts than others, for example the PUUV N-protein. Selection against recombinant proteins or peptides may favor the isolation of antibodies directed against linear epitopes. Previous studies have revealed the importance of using enriched preparations of the viral envelope proteins to enable the selection of neutralizing antibodies [Arikawa et al., 1989; Lundkvist et al., 1993]. By using rodent MAbs, bound to a solid-phase for affinity purification/selection of native G1/G2, it was possible to generate one neutralizing G2-specific Fab. Notably, no G1-specific Fab was isolated. Similarly, only G2-specific clones could be established when G1/G2-specific B cells were selected with magnetic beads coated with MAb-bound antigen [Lundkvist et al., 1993]. The results may indicate that there are fewer epitopes available on G1 as compared to G2, or that the G1-a epitope blocked by the capture MAb 5A2 constitutes a major antigenic site for the human antibody response. Another possible explanation is the higher IgG activity against G2, as indicated previously by the donor serum end-point titers [Lundkvist et al., 1993].

The finding that the isolated clones in this study were identical sequentially within the antibody binding regions CDR1-3, and also identical to the non-neutralizing PUUV G2-specific Fab puuA1 isolated

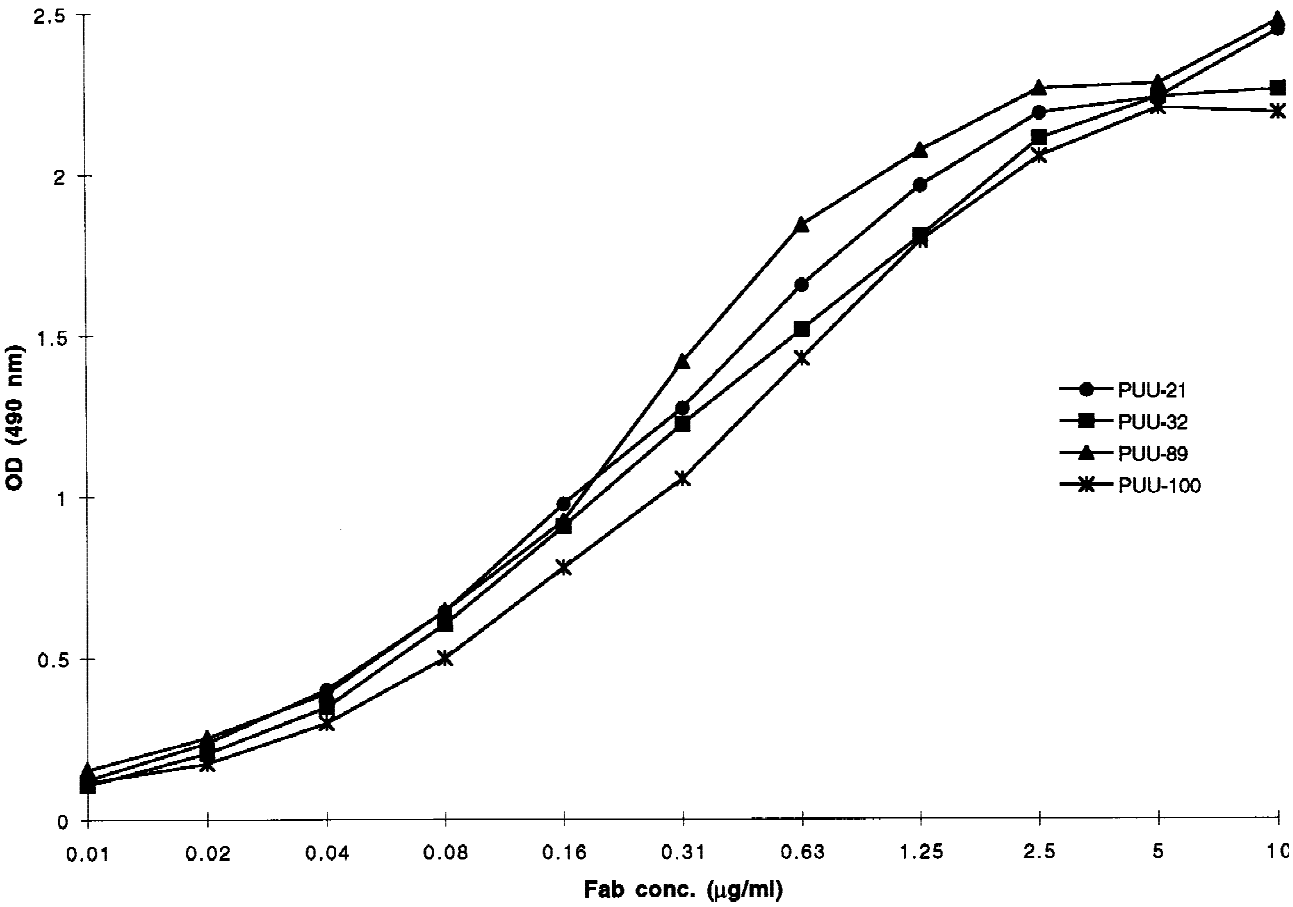

Fig. 1. ELISA titration of PUUV G2 Fabs against detergent-treated PUUV antigen.

TABLE III. Neutralizing Capacity of Human Fab Clones Against Different Strains of PUUV

		Virus strain					
		PUU Sotkamo		PUU Vindeln 83-L20		PUU Kazan-E6	
Fab	Concentration (μg/ml)	50%	80%	50%	80%	50%	80%
PUU-21	270	256[a]	16	4	4	4	4
PUU-32	240	256	64	4	4	4	<4
PUU-89	260	256	16	<4	<4	<4	<4
PUU-100	230	256	16	<4	<4	4	<4
Neg-7[b]	300	<2	<2	<2	<2	<2	<2

[a]Reciprocal neutralization titer.
[b]Neg-7 is an irrelevant negative Fab clone.

previously [Salonen et al., 1998] could be due to immunodominance. The occurrence of the same single G2 Fab from two independent experiments in two different laboratories is probably explained by the fact that the same combinatorial library was used on both occasions and that recognition of this epitope is highly dominant in the donor from which the RNA originated. The isolated clones are directed most probably to an epitope partially overlapping the immunodominant epitope recognized by the neutralizing G2-specific MAb 1C9 isolated from the same spleen.

It is not unlikely that certain clones dominate over others in the phage display expression system. Some clones could theoretically be expressed more easily with correct folding in *E. coli,* whereas others may have difficulties in achieving the right configuration and subsequent biological activity. Although Fab fragments are relatively small structures, consisting of two linked protein chains, conformational changes during expression could be relevant for their neutralizing capacity.

Our conclusion that all the Fab clones had identical heavy and light chain antigen binding sequences as did a non-neutralizing Fab described previously might be explained by small differences in treatment. A longer incubation time at low pH during affinity purification before neutralization of the pH, could result in biologi-

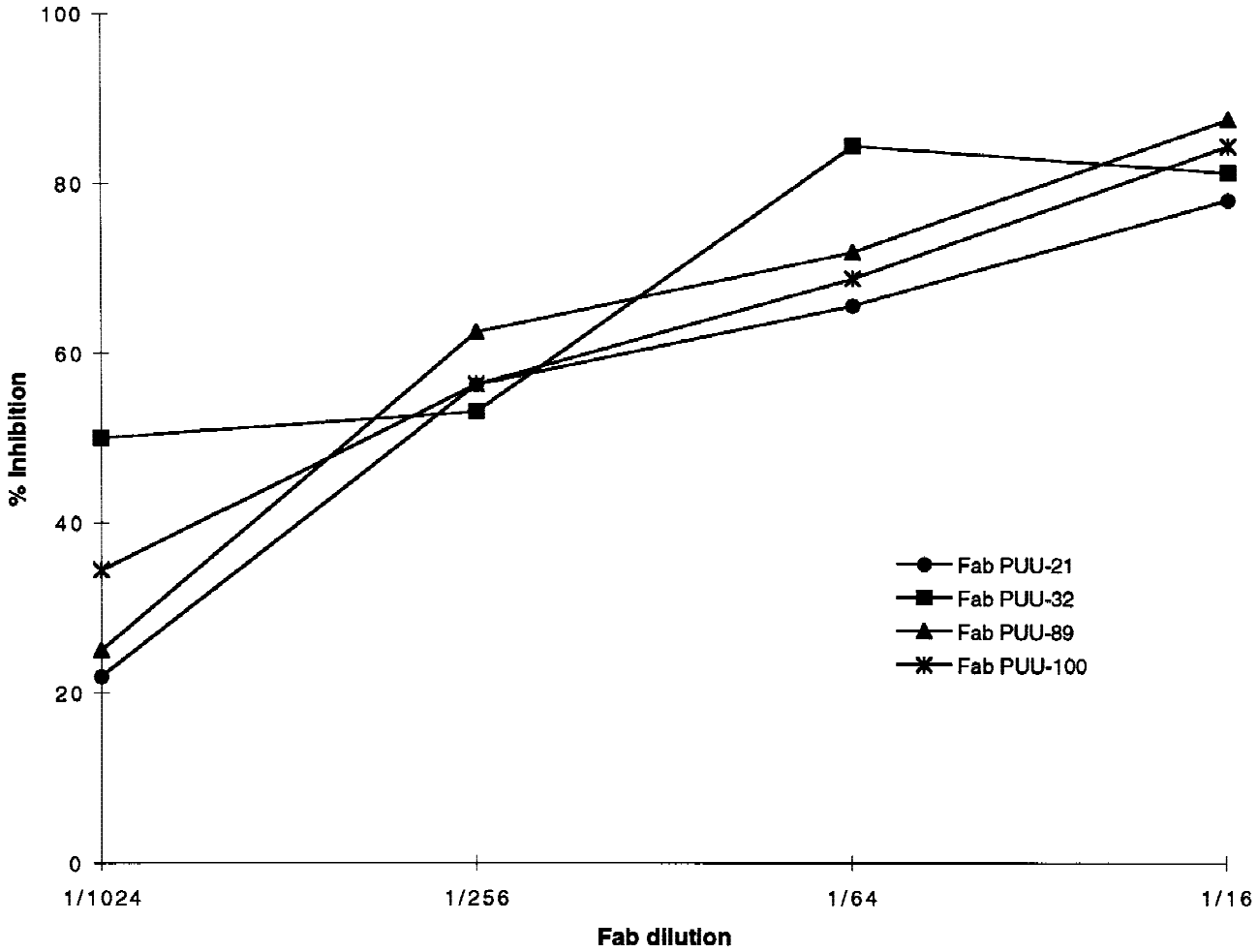

Fig. 2. Neutralization of PUUV by purified Fab fragments. Results shown are derived from the focus reduction neutralization test (FRNT) using the Sotkamo strain of PUUV; ≈80% neutralization was observed for Fab PUU-21 at 17 μg/ml, Fab PUU-32 at 4 μg/ml, Fab PUU-89 at 16 μg/ml and Fab PUU-100 at 14 μg/ml.

TABLE IV. Cross-Competition Between Fab Clones and MAbs for Binding to the PUUVG2 Protein Captured by the G2 Specific MAb 5B7

	Inhibiting antibody			
Antibody	PUUV Fabs	MAb 1C9	MAb 4G2	PUUV conv. serum*
PUUV Fabs	+++	+	NT	NT
MAb 1C9	–	+++	++	+
MAb 4G2	+	+++	NT	++

+++, >80% inhibition; ++, 40–80% inhibition; +, 1–40% inhibition; –, 0% inhibition; NT, not tested.
*PUUV convalescent serum drawn 4 years post infection.

cal differences, e.g. loss of virus neutralization activity; however, as all Fab clones reacted with the G2 antigen by ELISA, the affinity did not seem to be affected significantly.

Based on competition/inhibition experiments a new neutralizing epitope was mapped in the G2 protein. The epitope, designated G2-a3, was regarded as overlapping partially the G2-a2 epitope of the neutralizing MAb 1C9, because of the partial blocking apparent in competition experiments. In the previous work by Salonen et al. [1998] the same epitope was designated as G2-c; however, this was proposed because the Fab fragment puuA1 had no detectable neutralizing activity against any PUUV strain and complete competition experiments were not carried out. The G2-a3 epitope seem to be PUUV-specific because none of the purified Fabs reacted with any other hantavirus.

The specificity of the Fabs for the PUUV Sotkamo strain by FRNT as compared to the PUUV strains Vindeln 83-L20 and Kazan-E6 could be explained by the fact that PUUV Sotkamo was used in the initial panning of the Fabs. Some reactivity against the Swedish PUUV strain Vindeln 83-L20 could have been expected, however, as the donor (whose splenic cells were the source for RNA in the production of the combinatorial library) came from the northern part of Sweden where she also contracted, most likely, the infection. That no or only low Kazan-E6 neutralization was recorded could be due to sequence differences in the region of Fab recognition. The epitope of MAb 1C9, overlapping partially the epitope of the Fab isolated in this

TABLE V. Cross-Competition Between Fab Clones and PUUV Immune Sera for Binding to the PUUVG2 Protein Captured by the G2 Specific MAb 5B7

	Inhibiting sera/Fab/MAb						
Antibody	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	PUU-100	MAb 1C9
PUU-21	++	+++	+++	+++	+++	+++	+
PUU-32	++	+++	+++	+++	+++	+++	+
PUU-89	+++	+++	+++	+++	+++	+++	+
PUU-100	++	+++	+++	+++	+++	+++	+
MAb 1C9	–	++	+	+	+	–	+++

+++, >80% inhibition; ++, 40–80% inhibition; +, 1–40% inhibition; –, 0% inhibition.

study, has been mapped previously to amino acid 944 by an escape mutant [Hörling and Lundkvist, 1997]. Interestingly, comparison of the amino acid sequences in this region revealed two amino acid substitutions close to amino acid 944 in both PUUV strains Kazan-E6 and Vindeln 83-L20, as compared to PUUV strain Sotkamo (Fig. 3).

Knowledge concerning antibody inactivation of viral infections is important for understanding how passive immunization should be used for prevention or combat different virus infections in vivo, and to determine the nature of antibodies that future vaccines should aim to stimulate. Passive immunization with MAbs in challenge studies with rabies and respiratory syncytial virus (RSV) has revealed effective in vivo protection. [Enssle et al., 1991; Wyde et al., 1995]. Administration of murine MAbs for therapeutic purposes, however, is complicated by the fact that such molecules are recognized as non-self by the human immune system, and thus the development of strong human anti-mouse antibody responses potentially limits the usefulness of such MAbs. In addition, the half-life of mouse antibodies in the human circulation is generally short. Thus, the use of the combinatorial library technique, that offers an efficient route of obtaining human antibodies from an immune donor, is a promising alternative as has been reported previously for RSV and HIV [Crowe et al., 1994; Burton et al., 1994].

Fab antibody fragments and the respective whole IgG molecules could also be important tools for the study of the mechanisms of hantavirus neutralization. Does the antibody inhibit virus binding to the cell or does it interfere with fusion and entry or even at later stages? From this point of view recent knowledge of cellular receptor(s) for the virus is of great importance [Gavrilovskaya et al., 1999]. It is presumed that Fab neutralization is not accomplished by cross-linking or aggregation of viral proteins; however, several Fabs could block binding, fusion or entry either sterically or via binding to epitopes important for the different steps in viral replication. Inhibition by binding to viral attachment sites is a relative rare cause of neutralization, as neutralization sites are commonly distinct from attachment sites [Dimmock, 1993]. Antibodies can also be regarded as inhibitors that allow investigation of the early processes of infection, and knowledge of how they act may lead to the development of antivirals that mimic their mode of action.

This study contributes to the knowledge concerning

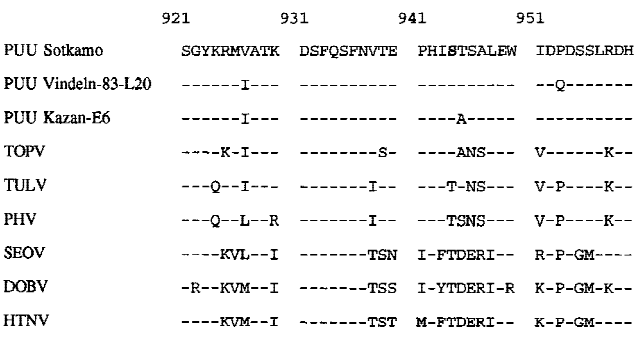

Fig. 3. Amino acid comparison of different hantaviruses in the binding region of the human anti-PUUV G2 MAb 1C9. The 1C9 escape mutant-specific mutation site at position 944 is in bold. PUUV strain Sotkamo [Hörling and Lundkvist, 1997], PUUV strain Vindeln 83-L20 (accession number Z49214), PUUV strain Kazan-E6 (Z84205), TOPV [Vapalahti et al., 1999], TULV strain Tula-MA76-1987 (Z69991), PHV strain PH-1 (X55128), SEOV strain 80-39 (S47716), DOBV (L33685), HTNV strain 76–118 (M14627).

which regions of the G2 protein of PUUV are important for the protective humoral immune response in humans. Furthermore, these Fabs will allow us to study further the mechanisms of neutralization of hantaviruses, and finally, reconstitution of Fabs to whole IgG molecules may be a strategy that could be used for future passive immunotherapy against PUUV infection.

ACKNOWLEDGMENTS

We thank Mariethe Ehnlund for expert technical assistance, Connie Schmaljohn for providing SN virus, and Robert Harris for critical reading of the manuscript.

REFERENCES

Antoniadis A, Stylianakis A, Papa A, Alexiou-Daniel S, Lampropoulos A, Nichol ST, Peters CJ, Spiropoulou CF. 1996. Direct genetic detection of Dobrava virus in Greek and Albanian patients with hemorrhagic fever with renal syndrome. J Infect Dis 174:407–410.

Arikawa J, Schmaljohn AL, Dalrymple JM, Schmaljohn CS. 1989. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. J Gen Virol 70:615–624.

Arikawa J, Yao JS, Yoshimatsu K, Takashima I, Hashimoto N. 1992. Protective role of antigenic sites on the envelope protein of Hantaan virus defined by monoclonal antibodies. Arch Virol 126:271–281.

Avsic-Zupanc T, Xiao SY, Stojanovic R, Gligic A, van der Groen G, LeDuc JW. 1992. Characterization of Dobrava virus: a Hantavirus from Slovenia, Yugoslavia. J Med Virol 38:132–137.

Barbas CF III, Kang AS, Lerner RA, Benkovic SJ. 1991. Assembly of

combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci USA 88:7978–7982.

Burton DR, Barbas CF III, Persson MAA, Koenig S, Chanock RM, Lerner RA. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc Natl Acad Sci USA 88:10134–10137.

Burton DR, Barbas CF III. 1994. Human antibodies from combinatorial libraries. Adv Immunol 57:191–280.

Burton DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, Parren PW, Sawyer LS, Hendry RM, Dunlop N, Nara PL, Lamacchia M, Garratty E, Stiehm ER, Bryson YJ, Cao Y, Moore JP, Ho DD, Barbas CF III. 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 266:1024–1027.

Chu YK, Rossi C, Leduc JW, Lee HW, Schmaljohn CS, Dalrymple JM. 1994. Serological relationships among viruses in the Hantavirus genus, family Bunyaviridae. Virology 198:196–204.

Crowe JE Jr., Murphy BR, Chanock RM, Williamson RA, Barbas CF III, Burton DR. 1994. Recombinant human respiratory syncytial virus (RSV) monoclonal antibody Fab is effective therapeutically when introduced directly into the lungs of RSV-infected mice. Proc Natl Acad Sci USA 91:1386–1390.

Dimmock NJ. 1993. Neutralization of animal viruses. Curr Top Microbiol Immunol 183:1–149.

Enssle K, Kurrle R, Kohler R, Muller H, Kanzy EJ, Hilfenhaus J, Seiler FR. 1991. A rabies-specific human monoclonal antibody that protects mice against lethal rabies. Hybridoma 10:547–556.

Gavrilovskaya IN, Brown EJ, Ginsberg MH, Mackow ER. 1999. Cellular entry of Hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by beta3 integrins. J Virol 73:3951–3959.

Heiskanen T, Lundkvist Å, Vaheri A, Lankinen H. 1997. Phage-displayed peptide targeting on the Puumala hantavirus neutralization site. J Virol 71:3879–3885.

Hörling J, Cheng Y, Plyusnin A, Persson K, Lehvaslaiho H, Vaheri A, Niklasson B, Lundkvist Å. 1995. Nucleotide and deduced amino acid sequences of the M and S genome segments of a Swedish Puumala virus isolate. Virus Res 39:321–330.

Hörling J, Chizhikov V, Lundkvist Å, Jonsson M, Ivanov L, Dekonenko A, Niklasson B, Dzagurova T, Peters CJ, Tkachenko E, Nichol S. 1996. Khabarovsk virus: a phylogenetically and serologically distinct hantavirus isolated from Microtus fortis trapped in far-east Russia. J Gen Virol 77:687–694.

Hörling J, Lundkvist Å. 1997. Single amino acid substitutions in Puumala virus envelope glycoproteins G1 and G2 eliminate important neutralization epitopes. Virus Res 48:89–100.

Kanerva M, Paakkala A, Mustonen J, Paakkala T, Lahtela J, Pasternack A. 1996. Pulmonary involvement in nephropathia epidemica: radiological findings and their clinical correlations. Clin Nephrol 46:369–378.

Lee HW, Lee PW, Johnson KM. 1978. Isolation of the etiologic agent of Korean Hemorrhagic fever. J Infect Dis 137:298–308.

Lee HW, Baek LJ, Johnson KM. 1982. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. J Infect Dis 146:638–644.

Lee PW, Amyx HL, Gajdusek DC, Yanagihara RT, Goldgaber D, Gibbs CJ Jr. 1982. New hemorrhagic fever with renal syndrome-related virus in rodents in the United States. Lancet 2:1405.

Liang M, Guttieri M, Lundkvist Å, Schmaljohn C. 1997. Baculovirus expression of a human G2-specific, neutralizing IgG monoclonal antibody to Puumala virus. Virology 235:252–260.

Lopez N, Padula P, Rossi C, Lazaro ME, Franze-Fernandez MT. 1996. Genetic identification of a new hantavirus causing severe pulmonary syndrome in Argentina. Virology 220:223–226.

Lundkvist Å, Fatouros A, Niklasson B. 1991. Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. J Gen Virol 72:2097–2103.

Lundkvist Å, Hörling J, Athlin L, Rosén A, Niklasson B. 1993. Neutralizing human monoclonal antibodies against Puumala virus, causative agent of nephropathia epidemica: a novel method using antigen-coated magnetic beads for specific B cell isolation. J Gen Virol 74:1303–1310.

Lundkvist Å, Hukic M, Hörling J, Gilljam M, Nichol S, Niklasson B. 1997a. Puumala and Dobrava viruses cause hemorrhagic fever with renal syndrome in Bosnia-Herzegovina: evidence of highly cross-neutralizing antibody responses in early patient sera. J Med Virol 53:51–59.

Lundkvist Å, Cheng Y, Brus Sjölander K, Niklasson B, Vaheri A, Plyusnin A. 1997b. Cell culture adaptation of Puumala hantavirus changes the infectivity for its natural reservoir, *Clethrionomys glareolus*, and leads to accumulation of mutants with altered genomic RNA S segment. J Virol 71:9515–9523.

Lundkvist Å, Niklasson B. 1992. Bank vole monoclonal antibodies against Puumala virus envelope glycoproteins: identification of epitopes involved in neutralization. Arch Virol 126:93–105.

Lundkvist Å, Niklasson B. 1994. Haemorrhagic fever with renal syndrome and other hantavirus infections. Rev Med Virol 4:177–184.

McCafferty J, Griffiths AD, Winter G, Chiswell DJ. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348:552–554.

Nichol ST, Ksiazek TG, Rollin PE, Peters CJ. 1996. Hantavirus pulmonary syndrome and newly described hantaviruses in the United States. In: Elliot R. editor. The Bunyaviridae. New York: Plenum Press. p 269–280.

Persson MAA, Caothien RH, Burton DR. 1991. Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. Proc Natl Acad Sci USA 88:2432–2436.

Plyusnin A, Vapalahti O, Lankinen H, Lehvaslaiho H, Apekina N, Myasnikov Y, Kallio-Kokko H, Henttonen H, Lundkvist Å, Brummer-Korvenkontio M, Gavrilovskaya I, Vaheri A. 1994. Tula virus: a newly detected hantavirus carried by European common voles. J Virol 68:7833–7839.

Plyusnin A, Vapalahti O, Vaheri A. 1996. Hantaviruses: genome structure, expression and evolution. J Gen Virol 77:2677–2687.

Salonen E-M, Parren PWHI, Graus YF, Lundkvist Å, Fisicaro P, Vapalahti O, Kallio-Kokko H, Vaheri A, Burton DR. 1998. Human recombinant Puumala virus antibodies: cross-reaction with other hantaviruses and use in diagnostics. J Gen Virol 79:659–665.

Samuelsson A, Chiodi F, Öhman P, Putkonen P, Norrby E, Persson MAA. 1995. Chimeric macaque/human Fab molecules neutralize Simian Immunodeficiency Virus. Virology 207:495–502.

Schmaljohn A, Li D, Negley D, Bressler D, Turell M, Korch G, Ascher M, Schmaljohn C. 1995. Isolation and initial characterization of a newfound hantavirus from California. Virology 206:963–972.

Schmaljohn CS, Chu YK, Schmaljohn AL, Dalrymple JM. 1990. Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. J Virol 64:3162–3170.

Schmaljohn CS. 1996. Molecular biology of hantaviruses. In: Elliott R, editor. The Bunyaviridae. New York: Plenum Press. p 63–90.

Vapalahti O, Kallio-Kokko H, Salonen E-M, Brummer-Korvenkontio M, Vaheri A. 1992. Cloning and sequencing of Puumala virus Sotkamo strain S and M RNA segments: evidence for strain variation in hantaviruses and expression of the nucleocapsid protein. J Gen Virol 73:829–838.

Vapalahti O, Lundkvist Å, Kukkonen SK, Cheng Y, Gilljam M, Kanerva M, Manni T, Pejcoch M, Niemimaa J, Kaikusalo A, Henttonen H, Vaheri A, Plyusnin A. 1996. Isolation and characterization of Tula virus, a distinct serotype in the genus Hantavirus, family Bunyaviridae. J Gen Virol 77:3063–3067.

Vapalahti O, Lundkvist Å, Fedorov V, Conroy CJ, Hirvonen S, Plyusnina A, Nemirov K, Fredga K, Cook JA, Niemimaa J, Kaikusalo A, Henttonen H, Vaheri A, Plyusnin A. 1999. Isolation and characterization of a Hantavirus from *Lemmus sibericus*: evidence for host-switch during hantavirus evolution. J Virol 73:5586–5592.

Wyde PR, Moore DK, Hepburn T, Silverman CL, Porter TG, Gross M, Taylor G, Demuth SG, Dillon SB. 1995. Evaluation of the protective efficacy of reshaped human monoclonal antibody RSHZ19 against respiratory syncytial virus in cotton rats. Pediatr Res 38: 543–550.

Zhang XK, Takashima I, Hashimoto N. 1989. Characteristics of passive immunity against hantavirus infection in rats. Arch Virol 105:235–246.